REMARKS

Claims 82-83 have been canceled without prejudice. Claims 1 and 59 have been amended for greater clarity. New claims 92-93 have been added to include the subject matter which is canceled from claims 1 and 59. Support for the claim amendments and new claims can be found throughout the specification (e.g., page 32, lines 16-21; and Tables 6-7 on pages 98-103) and original claims (e.g., claim 17). No new matter has been introduced and no new issue has been raised. The amendments have been made solely to expedite allowance. Applicants reserve the right to pursue claims of similar or differing scope in the future.

Applicants note with appreciation that the Examiner has withdrawn the previous rejections under 35 U.S.C. § 102(b) or 35 U.S.C. § 103(a) (citing Bennett et al., Khvorova et al., or Robbins et al.) as well as the rejection under 35 U.S.C. § 103(a) (citing Miyagishi et al., Promega, and Khvorova et al.). However, the Examiner has cited new art to raise the rejection below.

Applicants respectfully request reconsideration in view of the following remarks. Issues raised by the Examiner will be addressed below in the order they appear in the prior Office Action.

Claim Rejections under 35 USC § 103(a)

Claims 1, 5, 7-11, 14, 16, and 59 are rejected under 35 U.S.C. § 103(a) as being obvious over Stephenson et al. (BMC Mol Biol, 2001, 2:1-19), Bennett et al. (J. Biol chem, 1994, 268:14211-18), Taylor et al. (DDT, 1999, 4:562-67), Baracchini et al. (US Patent NO. 5801154), and Tang et al. (Nucl Acids Res, 1993, 21:2729-35). Applicants respectfully traverse this rejection.

According to the Examination Guidelines for Determining Obviousness Under 35 U.S.C. 103 In View of the Supreme Court Decision in *KSR International Co. v. Teleflex Inc.* (Federal Register Vol. 72, No. 195 at pages 57,526-57,535) (effective October 10, 2007) ("the Guidelines"), a § 103 claim rejection based on a purported teaching, suggestion or motivation to combine prior art references to arrive at the claimed invention must support a conclusion of obviousness by including: (1) a finding that there was some teaching, suggestion or motivation to modify or combine the cited references; (2) a finding that there was a reasonable expectation of success; and (3) whatever

additional findings based on the *Graham* factual inquiries may be necessary in view of the specific facts. In this case, Applicants submit that the Examiner has not satisfied the requirement of establishing a *prima facie* case of obviousness against independent claims 1 and 59.

Independent claims 1 and 59 as amended are both directed to an isolated nucleic acid compound between 20 and 35 nucleotides in length, which is 100% complementary to a region of an EphB4 transcript sequence set forth in SEQ ID NO: 392, wherein the nucleic acid compound decreases expression of EphB4 in a cell, and wherein the nucleic acid compound is an antisense nucleic acid compound comprising one or more modified backbone or base moieties. Applicants emphasize that the claimed EphB4 antisense compound is functionally defined by its ability to decreases expression of EphB4 in a cell.

Stephenson et al. merely describe that the EphB4 gene expression was increased in colon cancer cells and the EphB4 gene could be a potential candidate marker (see, e.g., the abstract and page 5, left column). Although Stephenson et al. suggest "targeted disruption of the EphB4 gene in colon cancer cells" (page 7, left column), Stephenson et al. do **not** teach use of antisense compounds, let alone an antisense compound which decreases expression of EphB4 in a cell. In fact, one of skill in the art would know that "targeted disruption of the EphB4 gene" as disclosed by Stephenson et al. is entirely different from the antisense technology. Indeed, it was well known in the art that antisense compounds do not disrupt a gene. Rather, antisense compounds inhibit expression of a gene.

Contrary to the Examiner's assertion, none of the other cited references bridge the gap between the claimed invention and Stephenson et al. Bennett et al. merely disclose that expression of the EphB4 gene was detected in several malignant cells lines (see, e.g., the abstract and Figure 5). However, Bennett et al. do **not** teach or suggest that EphB4 gene expression should be reduced, let alone the use of an antisense compound which decreases expression of EphB4 in a cell. Taylor et al., Baracchini et al., and Tang et al. merely teach antisense technology in general. Each of these three cited references does **not** teach how to make or modify an antisense compound targeting the EphB4 gene, let alone an antisense compound which decreases expression of EphB4 in a cell. Thus, even if Stephenson et al. are to be combined with Bennett et al., Taylor et al., Baracchini et al., and

Tang et al., the combination still fails to teach all the limitations of independent claim 1 and 59 (e.g., an antisense compound between 20 and 35 nucleotides in length, which is 100% complementary to a region of an EphB4 transcript sequence set forth in SEQ ID NO: 392, wherein the nucleic acid compound decreases expression of EphB4 in a cell).

Furthermore, Applicants submit that a skilled artisan would **not** have had a reasonable expectation of success even if these references were combined, given the state of the art at the time of the invention. It was well known in the art that the activity of antisense oligonucleotides is *unpredictable within cells*. See, e.g., Summerton and Weller (1997) Antisense & Nucleic Acid Drug Development. 7:187-195 (enclosed herewith as **Exhibit A**). As Summerton and Weller note, many technical problems may lead to the antisense inefficiency within cells, such as inadequate specificity, ineffective delivery into the proper subcellular compartment, and unpredictable activity within cells (see, e.g., the abstract). Each of the cited references fail to teach how to make or modify an antisense compound which decreases expression of EphB4 in a cell as recited in claims 1 and 59. Indeed, the specification discloses that not all of the antisense compounds can effectively decrease expression of EphB4 in cells (e.g., Table 6 on pages 98-101; and Figure 31D). In view of the unpredictable activity of antisense compounds within cells and the lack of guidance on how to make or modify an antisense compound which decreases expression of EphB4 in a cell, a skilled artisan could not predict that the antisense compound as recited in claim 1 and 59 would be successfully made.

In addition, the alleged combination fails to provide any suggestion or motivation for a skilled artisan to make an antisense compound as recited in claims 1 and 59. Stephenson et al. teach "targeted **disruption** of the EphB4 **gene** in colon cancer cells" (page 7, left column, emphasis added). One of skill in the art would know that "targeted disruption of the EphB4 gene" is entirely different from the antisense technology since antisense compounds do not disrupt a gene. As such, Stephenson et al. effectively teach away from the claimed invention. In the absence of any evidence that antisense compounds need to be produced against the EphB4 gene, one of ordinary skill in the art would have had no motivation to make the antisense compounds such as those recited in claims 1 and 59.

In sum, there is no reasonable expectation of success for a skilled artisan to arriving at the claimed antisense compounds and one of skill in the art would not have been motivated to design the antisense compounds as recited in the pending claims. Applicants note that the Examiner has not has not satisfied the requirement of establishing a *prima facie* case of obviousness.

In view of the above, Applicants submit that independent claims 1 and 59 as well as their dependent claims are not obvious over the cited references. Reconsideration and withdrawal of the rejection are respectfully requested.

CONCLUSION

In view of the above amendment, applicant believes the pending application is in condition for allowance. Applicant believes no fee is due with this response. However, if a fee is due, please charge our Deposit Account No. 18-1945, under Order No. **VASG-P01-001** from which the undersigned is authorized to draw.

Dated: October 17, 2008

Respectfully submitted,

By _____

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ANTISENSE & NUCLEIC ACID DRUG DEVELOPMENT 7:187–195 (1997)
Mary Ann Liebert, Inc.

Exhibit A

Review Article

Morpholino Antisense Oligomers: Design, Preparation, and Properties

JAMES SUMMERTON and DWIGHT WELLER

ABSTRACT

Antisense promised major advances in treating a broad range of intractable diseases, but in recent years progress has been stymied by technical problems, most notably inadequate specificity, ineffective delivery into the proper subcellular compartment, and unpredictable activity within cells. Herein is an overview of the design, preparation, and properties of Morpholino oligos, a novel antisense structural type that solves the sequence specificity problem and provides high and predictable activity in cells. Morpholino oligos also exhibit little or no nonantisense activity, afford good water solubility, are immune to nucleases, and are designed to have low production costs.

INTRODUCTION

OLIGONUCLEOTIDES, OLIGONUCLEOTIDE ANALOGS, and other sequence-specific binding polymers designed to block translation of selected messenger RNAs (the sense strand) are commonly called "antisense oligos." Development of such oligos for therapeutic applications, which constitutes the epitome of rational drug design, entails selecting a target genetic sequence unique and critical to the pathogen or pathogenic state one wishes to treat. One then assembles an oligomer of genetic bases (adenine, cytosine, guanine, and thymine or uracil) complementary to that selected sequence. When such an antisense oligo binds to its targeted disease-causing sequence, it can inactivate that target and thereby alleviate the disease.

Antisense oligos offer the prospect of safe and effective therapeutics for a broad range of intractable diseases. Nonetheless, developing therapeutics that function by a true antisense mechanism presents a number of forbidding challenges. The oligos should achieve adequate efficacy at a concentration attainable within the cells of the patient. They should inhibit their selected target sequences without concomitant attack on any other sequences in the patient's pool of approximately 200 million bases of unique-sequence RNA. They should be stable in extracellular compartments and within cells. They must be deliverable into the cellular compartment(s) containing their targeted sequences. They should be adequately soluble in aqueous solution. They should exhibit little or no toxicity at therapeutic concentrations. Finally, they should be affordable, reflecting the increasing awareness that health care, even for life-threatening conditions, should not expend an excessive portion of society's resources.

First-generation antisense oligos comprised natural genetic material (Belikova et al., 1967; Zamecnik and Stephenson, 1978; Summerton, 1979) and often contained crosslinking agents for binding their targets irreversibly (Summerton and Bartlett, 1978a,b). As the design challenges became more fully appreciated, a number of nonnatural antisense structural types (Fig. 1) were developed in an effort to improve efficacy, stability, and delivery. Of particular note are the early non-ionic DNA analogs developed by Miller and Ts'o, including phosphotriester-linked DNA (Miller, 1989a) and methylphosphonate-linked DNA (Miller, 1989b). Other nucleic acid analogs of note include carbamate-linked DNA (Stirchak et al., 1987), phosphorothioate-linked DNA (Stein and Cohen, 1989), phosphoroamidate-linked DNA (Froehler et al., 1988), α-DNA (Rayner et al., 1989), and 2′-O-methyl RNA (Shibahara et al., 1989). Figure 1B shows several novel antisense types that no longer resemble nucleic acids. These oligos contain acyclic backbone moieties, including nylon (Weller et al. 1991; Huang et al., 1991), the exceptionally high-affinity peptide nucleic acids (PNAs) (Egholm et al., 1992), and related types (Summerton and Weller, 1993a).

Although each of these newer structural types provides one or more significant advantages over the first-generation oligos, none yet appear to provide the full combination of properties needed in antisense therapeutics for clinical applications.

ANTIVIRALS Inc., Corvallis, OR 97333.

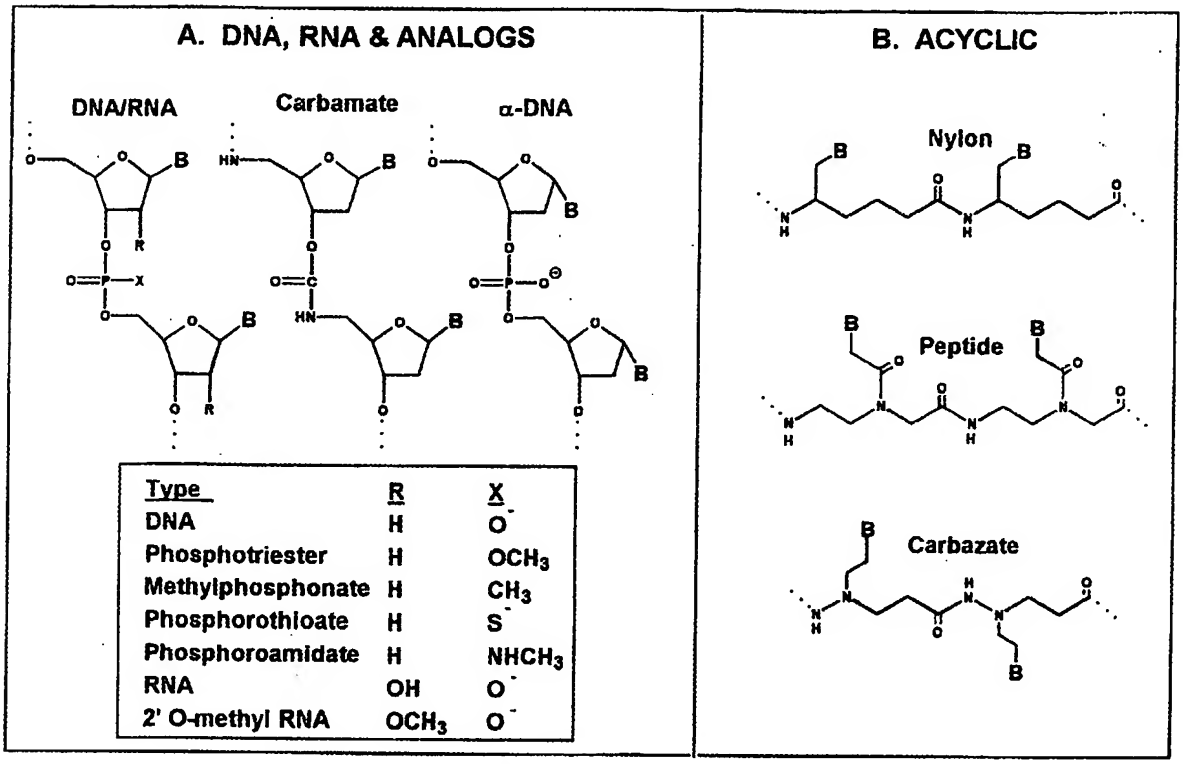

FIG. 1. Representative antisense structural types.

Herein we describe the design considerations used in developing a novel Morpholino structural type (Fig. 2), which affords antisense oligos having very high efficacy and specificity, immunity to nucleases, good aqueous solubility, and low production costs.

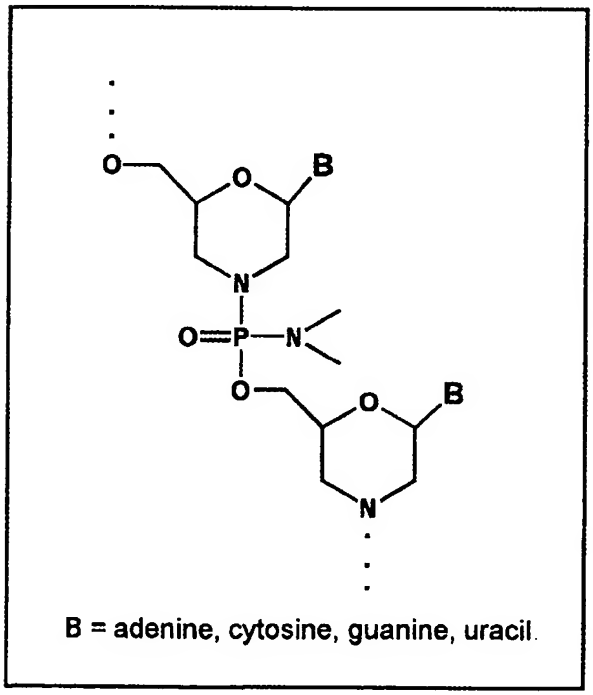

FIG. 2. Morpholino oligo structure.

DESIGN

Backbone structure

A dominant consideration in the design of most antisense oligos has been to devise a structure that provides resistance to nucleases while still resembling natural nucleic acids as closely as possible. This conservative approach has spawned a number of DNA analogs (Fig. 1A) that may be unduly expensive for routine applications requiring systemic delivery. The high cost of DNA and its analogs is due in part to the low abundance of DNA in production-scale source material and the difficulty in cleaving DNA to the deoxyribonucleosides required for preparing DNA analogs. An additional factor in their high cost is the complexity and expense of coupling to hydroxyls, required in forming the phosphoester intersubunit linkages of most DNA analogs.

Rather than trying to solve inherent cost problems after a structural type has been developed, a better approach is to incorporate fundamental cost advantages in the initial structural design stage. Following this strategy, we reasoned that more affordable antisense oligos might be possible if inexpensive ribonucleosides could be exploited as starting material. The order-of-magnitude cost advantage of ribonucleosides relative to deoxyribonucleosides (Summerton, 1992) derives from the sixfold greater abundance of RNA relative to DNA in production-scale source material (e.g., yeast cake) and the ease of cleaving

RNA to its component ribonucleosides. It is noteworthy that ribonucleosides are now directly available from special excreting strains of yeast, further reducing their cost. However, the use of ribonucleosides for preparation of RNA and RNA analogs presents two serious problems. First, during oligo assembly, one must selectively couple either the 2′ or the 3′ hydroxyl. This is typically achieved in a relatively expensive manner by selectively masking the 2′ hydroxyl with a cleavable or noncleavable moiety. The second problem is that coupling to the 3′ hydroxyl of the riboside is even more difficult and expensive than the corresponding coupling of deoxyribonucleosides.

We envisioned that these problems could be circumvented by converting the riboside moiety to a morpholine moiety (Stirchak et al., 1989; Summerton, 1990) (Fig. 3). Although oligomers assembled from such Morpholino subunits differ substantially from DNA, RNA, and analogs thereof, our initial modeling studies carried out in 1985 suggested that such novel Morpholino-based oligomers might constitute useful and highly cost-effective antisense agents. The simple and inexpensive ribose to morpholine conversion shown in Figure 3 replaces two poor nucleophiles (the 2′ and 3′ hydroxyls) with a single good nucleophile (the morpholine nitrogen) and allows oligo assembly via simple and efficient coupling to the morpholine nitrogen without the expensive catalysts and postcoupling oxidation steps required in the production of most DNA-like antisense oligos. It is noteworthy that in spite of the relatively low nucleophilicity of the morpholine nitrogen ($pK_a = 5.75$), we still typically achieve coupling efficiencies of 99.7% without using catalysts.

Intersubunit linkage

We have assessed a substantial number of intersubunit linkage types, including the carbonyl, sulfonyl, and phosphoryl linkages (Fig. 4) (Summerton and Weller, 1991, 1993a,b; Stirchak et al., 1989). Although Morpholino oligos containing a number of such linkages provide effective binding to targeted genetic sequences, consideration of cost and ease of synthesis, chemical stability, aqueous solubility, and affinity and homogeneity of binding to RNA led us to focus on the phosphorodiamidate shown in Figure 2 as our principle linkage type for oligos targeted against single-stranded RNA sequences. These non-ionic phosphorodiamidate-linked Morpholino oligos exhibit quite good binding to complementary nucleic acids, particularly RNA sequences. Table 1 compares the temperature of melting (T_m) values at physiologic salt concentration for identical-sequence 20-mer oligos of three different antisense structural types paired with their complementary RNA. As seen in Table 1, RNA binding affinity is lowest for the phosphorothioate-linked DNA (S-DNA), appreciably higher for DNA, and highest for the Morpholino oligo.

PREPARATION

Oligo assembly

Although phosphorodiamidate-linked Morpholino oligos can be assembled by a variety of methods, one relatively simple method that has proved effective (Summerton and Weller, 1993b) entails protection and activation of the Morpholino subunit (Fig. 5A). The activated subunits can be stored at low temperatures for many months without significant breakdown. Whereas they are relatively resistant to hydrolysis, they react rapidly ($T_{1/2}$ of 1–2 minutes) with the morpholine nitrogen of growing chains on a 1% crosslinked polystyrene synthesis support loaded at 500 μM/g of resin, with coupling efficiencies typically about 99.7%. A preferred oligo assembly cycle (Summerton and Weller, 1993b) is shown in Figure 5B. It is noteworthy that in large-scale syntheses, excess activated subunit used in the coupling step can be recovered and reused, effecting a further substantial reduction in production costs.

Because of cheaper starting materials and simpler, more efficient oligo assembly, we estimate that in large-scale production, the cost of these Morpholino antisense oligos will be at least an order of magnitude lower than the cost of corresponding DNA analogs (Summerton, 1992).

PROPERTIES

Solubility

For an antisense oligo to have effective access to its target sequence within the cytoplasm of a cell, the oligo should show reasonable water solubility. Good water solubility may also prove essential for systemic delivery of antisense oligos. Conventional wisdom in the antisense field is that non-ionic antisense oligos invariably show poor water solubility. In this regard, it is interesting that a Morpholino dimer containing a rigid carbamate linkage shows little or no base stacking (Kang et al., 1992), and in the absence of special solubilizing groups, Morpholino oligomers containing such carbamate linkages are quite insoluble in aqueous solutions (Stirchak et al., 1989). In contrast, phosphorodiamidate-linked Morpholino oligos of the type shown in Figure 2 show excellent base stacking (Kang et al., 1992) and are several orders of magnitude more soluble in aqueous solutions. To illustrate the exceptional aqueous solu-

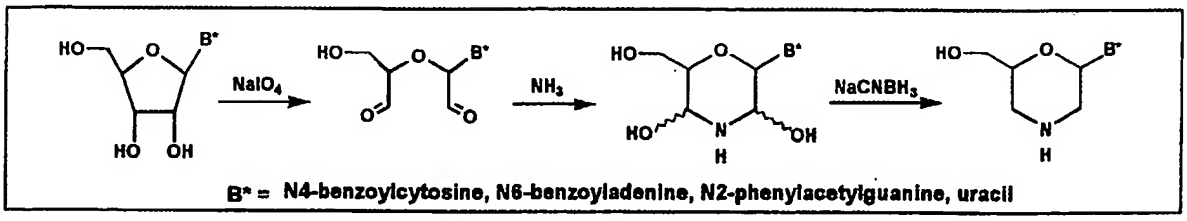

FIG. 3. Conversion of ribonucleoside to Morpholino subunit.

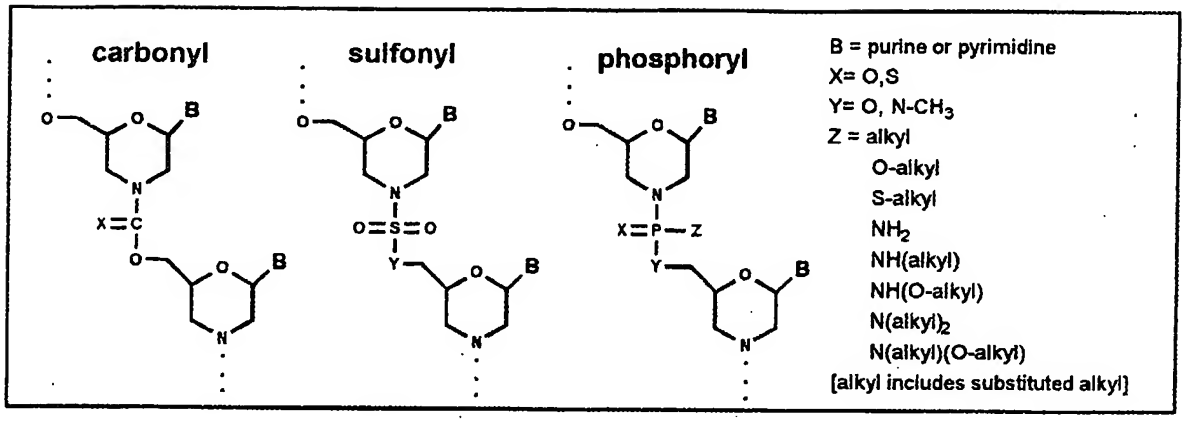

FIG. 4. Intersubunit linkage types for Morpholino oligos.

bility of Morpholino oligos of this type, we have dissolved 263 mg of a heteromeric 22-mer of the sequence 5′-GCUCGCA-GACUUGUUCCAUCAU in 1 ml of water (36 millimolal) at 20°C without reaching saturation.

We suggest that the poor water solubility of the carbamate-linked Morpholino oligos results at least in part from the difficulty of inserting the hydrophobic faces of the unstacked bases into an aqueous environment. In contrast, it seems likely that the excellent water solubility of the phosphorodiamidate-linked Morpholino oligos is a consequence of effective shielding of these hydrophobic faces from the polar solvent because of good stacking of the bases.

Biologic stability

To achieve reasonable efficacy, an antisense oligo should not be degraded rapidly either extracellularly or within cells. In this regard, it has been demonstrated that DNA and 2′-O-methyl RNA are rapidly degraded and phosphorothioate DNA is slowly degraded by nucleases in blood and within cells (Hoke et al., 1991; Morvan et al., 1993). Although resistance to nucleolytic degradation can be improved by adding special groups to the termini (Cazenave et al., 1987) or by incorporating a few nuclease-resistant intersubunit linkages near each end (Larrouy et al., 1992), we believe a better solution, on the basis of both function and cost, is to use a backbone structure that is inherently immune to a broad range of degradative enzymes present in the blood and within cells. A further advantage of using a backbone structure that is not degraded in the body is that it avoids concerns that modified nucleosides or nucleotides resulting from degradation of an antisense oligo might be toxic or might be incorporated into cellular genetic material and thereby lead to mutations or other undesired biologic effects.

In experiments detailed elsewhere (Hudziak et al., 1996), it is demonstrated that Morpholino phosphorodiamidate oligos of the type shown in Figure 2 are immune to a wide range of nucleases, including DNase I (an endonuclease that cleaves both single-stranded and double-stranded DNA), DNase II (cleaves between the 5′ oxygen and the phosphorus of DNA linkages), RNase A (cleaves on the 3′ side of pyrimidines), RNase T1 (cleaves on the 3′ side of guanines), nuclease P1 (cleaves single-stranded RNA and DNA), phosphodiesterase (3′ exonuclease for both RNA and DNA), Mung bean nuclease (cleaves single-stranded RNA and DNA), and benzonase (cleaves both single-stranded and double-stranded RNA and DNA, including linear, circular, and supercoiled). These Morpholino oligos have also been found to be immune to pronase E, proteinase K, and pig liver esterase, as well as degradative enzymes in serum and a liver homogenate.

TABLE 1. MELTING TEMPERATURES OF RNA/OLIGO DUPLEXES

RNA/S-DNA	68.5°C
RNA/DNA	77.3°C
RNA/Morpholino	81.3°C

Antisense efficacy

Because of the excellent RNA binding affinity of oligos of this phosphorodiamidate-linked Morpholino structural type, it seemed likely Morpholino oligos would be effective in blocking translation of their targeted mRNAs, and this has been found to be the case. In cell-free translation experiments using a sensitive luciferase reporter, we have demonstrated that a Morpholino oligo 25 subunits in length, in both the presence and absence of RNase H, inhibits its targeted mRNA somewhat better than the corresponding S-DNA oligo in the presence of added RNase H, with both showing good efficacy at concentrations of 10 nM and above. Representative translational inhibition results are shown in Figure 6 (Summerton et al., 1997). A similar comparison of Morpholino and S-DNA antisense oligos targeted against murine tumor necrosis factor-α (TNF-α) mRNA in a cell-free translation system also showed greater activity for the Morpholino oligos (Taylor et al., 1996).

Specificity

In the early days of antisense research, one of the most compelling arguments for antisense therapeutics was their promise of exquisite specificity for their targeted genetic sequences. However, as the most synthetically accessible antisense structural types (DNA and S-DNA) have come into broad use, it has become clear that these two structural types provide reasonable

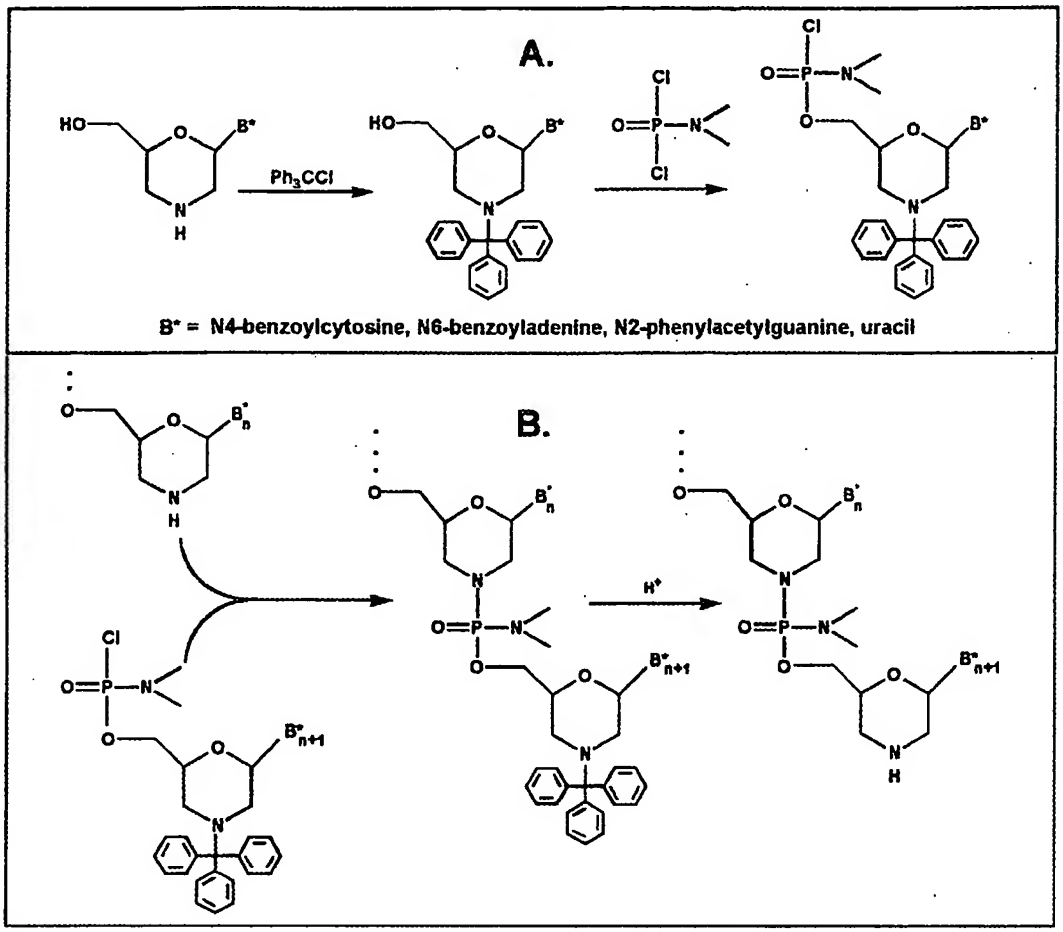

FIG. 5. Protection, activation, and coupling of Morpholino subunits.

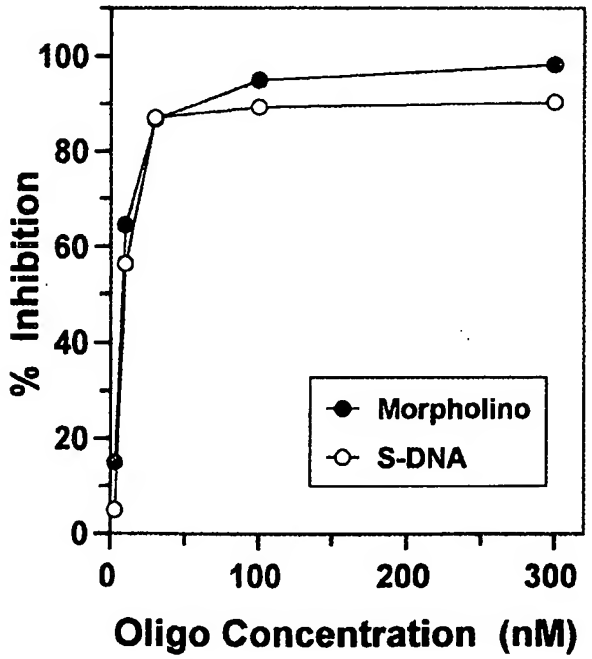

FIG. 6. Cell-free efficacy of Morpholino and S-DNA antisense oligos.

sequence specificity within only a very narrow concentration range (ANTIVIRALS Inc., 1993; Stein and Cheng, 1993).

We believe a key factor responsible for the low specificity of DNA and S-DNA oligos is their RNase H competency; that is, DNA and S-DNA form duplexes with complementary RNA that are readily cleaved by RNase H, an enzyme widely distributed in living organisms. The specificity problem arises because DNA/RNA and S-DNA/RNA duplexes as short as 5 base pairs in length are cleaved by RNase H (Crouch and Dirksen, 1982). Presuming about 6% of the genome is transcribed in higher animals, the patient's RNA pool will comprise about 200 million bases of unique-sequence RNA. With this level of sequence complexity, it is inevitable that antisense oligos will form many short transient duplexes with partially complementary nontarget sequences of inherent cellular RNAs. Cleavage of the RNA strand of such nontarget duplexes by endogenous RNase H (Larrouy et al., 1992; Cazenave et al., 1989) is expected to cause significant disruption of normal cellular translation. As this cleavage process releases the DNA or S-DNA in its original form, such oligos can continue the cycle of transiently pairing with additional nontarget cellular RNA sequences, cleavage of the RNA strand, and release of the antisense oligo. As a consequence, essentially every RNase H-competent oligo is expected to cleave hundreds to thousands of species of inherent cellular RNAs.

A second factor expected to contribute to superior specificity of Morpholino oligos relative to RNase H-competent types is that RNase H-independent oligos have far fewer potential targets in the inherent pool of cellular RNA. This is because most antisense structural types that do not support RNase H cleavage of their RNA targets have been found to be effective in blocking translation of their targeted mRNAs only when said oligos are complementary to sequences in the 5′ leader region of that mRNA or when they are targeted against other special sites, such as splice junctions and transport signals [e.g., methylphosphonate DNA (Walder and Walder, 1988), α-DNA (Rayner et al., 1989), 2′-O-methyl RNA (Shibahara et al., 1989), and Morpholino (Summerton et al., 1997)]. We estimate that such special targetable regions constitute on the order of 2%–5% of the sequeces in the cellular RNA pool. Presumably, this targeting limitation reflects the ability of ribosomes to displace essentially all antisense oligos during translocation down the coding region of mRNAs.

Because an antisense oligo that does not support RNase H cleavage cannot effectively block functioning of an RNA when said oligo is bound to sequences outside of special targetable regions, such an oligo only needs to distinguish its target sequence from those 2%–5% of the cellular RNA sequences comprising special targetable regions. In contrast, antisense oligos that form RNase H-cleavable duplexes with RNA can be effective when targeted essentially anywhere along an RNA transcript (Walder and Walder, 1988), presumably because RNase H cleavage at the target site of the antisense oligomer destroys the RNA, rendering moot the oligo displacement capability of translocating ribosomes. Accordingly, RNase H-competent oligos (DNA and S-DNA) face the much greater specificity challenge of distinguishing selected target sequences from essentially the entire pool of cellular RNA sequences. As a consequence, RNase H-independent oligos, such as Morpholinos, should enjoy a 20-fold to 50-fold advantage in sequence specificity because of this more than order-of-magnitude reduction in the number of inherent nontarget cellular sequences of any given length that they can inhibit.

A third factor compromising the specificity of S-DNA oligos is their promiscuous binding to proteins (Krieg and Stein, 1995), including components of the cell's replication, transcription, and translation machinery.

Given these factors expected to limit the sequence specificity of RNase H-competent antisense structural types, particularly S-DNA, we set out to compare sequence specificities of S-DNA and Morpholino antisense oligos. To this end, we carried out stringent specificity assays in a cell-free translation system using two oligos of each structural type (Summerton et al., 1997). In these experiments, one oligo was perfectly complementary to its target mRNA to provide a measure of the total inhibition afforded by that oligo type. The other oligo incorporated 4 mispairs to that same mRNA target sequence, with the longest run of perfect pairing comprising 10 contiguous base pairs, to provide an estimate of the low-specificity component of the inhibition. The difference between these two inhibition values at each concentration than provided a measure of the high-specificity component, which we denote as "sequence-specific inhibition."

Figure 7 (experimental as in Summerton et al., 1997) shows that the S-DNA oligo achieved reasonable efficacy at concentrations above about 10 nM, but the sequence-specific component of its inhibition dropped below 50% at concentrations of only 100 nM and higher. The corresponding Morpholino oligo achieved even better efficacy at 10 nM while maintaining good sequence specificity through 10,000 nM, the highest concentration tested. Thus, in this stringent test of specificity, the Morpholino oligo achieved highly effective and specific antisense activity over a concentration range more than two orders of magnitude greater than the concentration range wherein the corresponding S-DNA achieved reasonable efficacy and specificity.

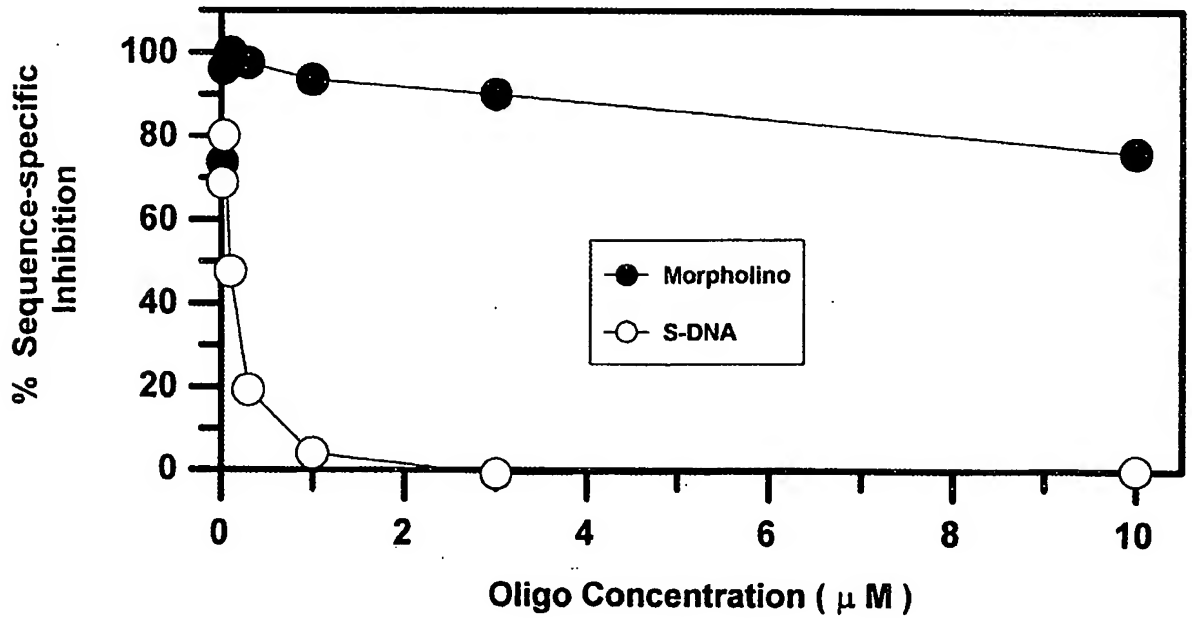

FIG. 7. Sequence specificity of Morpholino and S-DNA oligos.

Taylor et al., (1996) have reported that S-DNAs targeted against TNF-α mRNA showed very poor sequence specificity in a cell-free translation system, whereas the corresponding Morpholino oligos afforded good specificity over the full range tested.

Activity in cells

For effective biologic activity, an antisense oligo must gain entry into the cellular compartments where the target genetic sequence is synthesized, processed, and functions—specifically, the cytosol/nuclear compartment. Our experiments with fluorescent-tagged Morpholino oligos suggested that these oligos enter mammalian cells by what appears to be endocytosis, but they do not appear to subsequently cross the endosomal or lysosomal membrane into the cytosol, based both on visualization of fluorescent-tagged oligos and a functional assay employing a transfected plasmid (Partridge et al., 1996). This result is in agreement with limitations on uptake of antisense oligos reported by others. Specifically, a number of studies have been reported that suggest that in the absence of experimental manipulations that compromise the cell membrane, both polyanionic oligos [e.g., S-DNA (Wagner et al., 1993; Tonkinson and Stein, 1994) and 2′-O-methyl RNA (Oberhauser and Wagner, 1992)] and non-ionic oligos [e.g., methylphosphonate DNA (Shoji et al., 1991) and PNAs (Bonham et al., 1995)] enter cells primarily or exclusively by endocytosis. Further, a number of studies on a variety of antisense structural types indicate that most or all of the antisense oligo that gains entry by endocytosis does not subsequently traverse the endosomal or lysosomal membrane to enter in an intact form into the cytosol, where protein synthesis occurs (Oberhauser and Wagner, 1992; Shoji et al., 1991; Bonham et al., 1995).

However, we have found that antisense oligos can be easily delivered into cultured cells simply by passaging anchorage-dependent cells by the common procedure of scraping with a rubber policeman. This has been shown to achieve significant oligo entry into the cytosolic compartment if the oligo is present during the scraping (Partridge et al., 1996). Further, Morpholino oligos delivered into cells by such scrape loading show good activity and specificity therein, whereas corresponding S-DNA oligos (both antisense and control sequences) largely fail to inhibit their targets within scrape-loaded cells at concentrations up to 3 μM in the medium and instead are often stimulatory (Summerton et al., 1997). Figure 8 shows a comparison of the activities of representative Morpholino and S-DNA oligos in scrape-loaded cells (experimental as in Summerton et al., 1997).

Taylor et al. (1996) have also compared the activity of S-DNA and Morpholino antisense oligos in cultured cells. In their studies, the S-DNAs were delivered into mouse macrophage-like cells (RAW 264.7) using lipofectin. Both oligo types were targeted against TNF-α mRNA, and treated cells were assessed for inhibition of lipopolysaccharide-induced TNF-α production. In agreement with our in-cell results, Taylor et al. report that both the antisense and control S-DNAs stimulated instead of inhibitied TNF-α production, whereas the Morpholino antisense oligo, although poorly delivered into the cells, afforded significant and specific inhibition of TNF-α production.

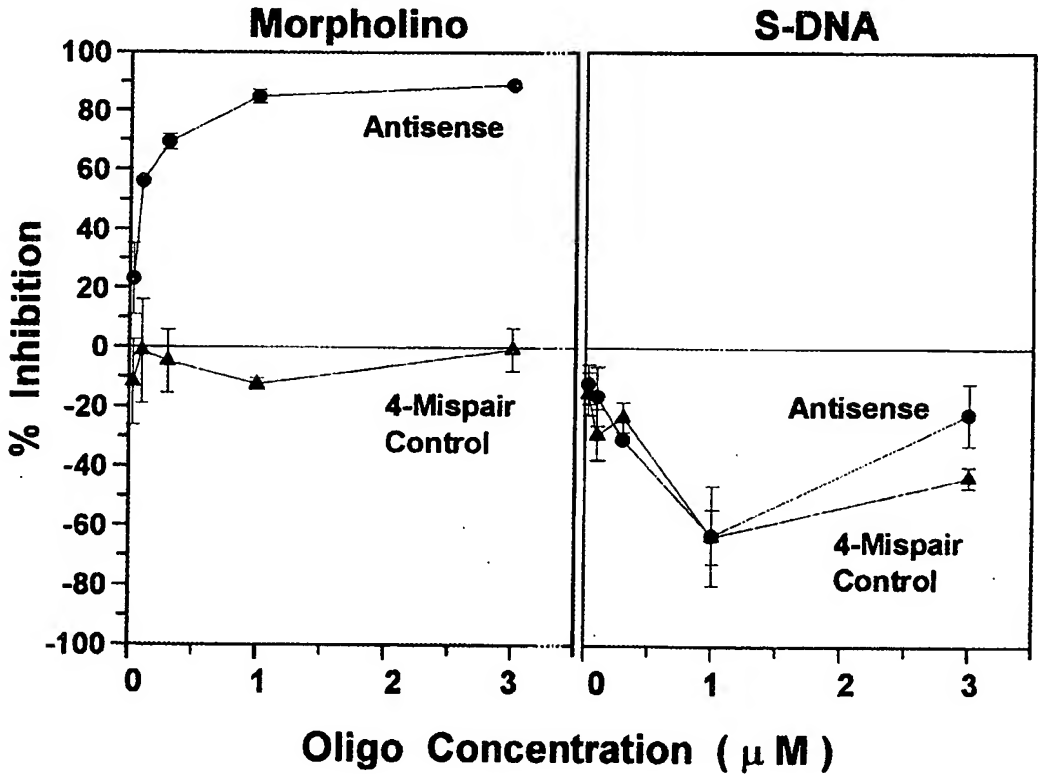

FIG. 8. In-cell activities of Morpholino and S-DNA oligos.

In vivo *properties*

To date, our principal efforts have focused on optimizing the Morpholino structural type and on studying the properties of Morpholino oligos at the biophysical level, in cell-free translation systems, and in cultured cells. In light of the promising results from those studies, we and several collaborators are now shifting our focus to *in vivo* studies.

A very preliminary ranging study was carried out to assess acute toxicity. In this study, a representative 20-mer Morpholino oligo in phosphate-buffered saline was injected intravenously into mice at doses ranging from 88 mg/kg to 700 mg/kg. No acute toxicity was seen at any of these doses. However, over a period of 2 weeks, an effect on body weight gain and ruffled coat was observed at the highest dose. Using the results from this ranging study, an extensive toxicity study has been initiated and will be the subject of a future report.

In addition, a variety of efficacy studies in mice and rats are in progress to assess the possible use of Morpholino oligos for therapeutic applications. We are also investigating possible methods for improving the delivery of these oligos into the cytosol/nuclear compartment of cells *in vivo*.

DISCUSSION

Morpholino oligos meet key requirements for safe, effective, and affordable antisense therapeutics, including high efficacy at low nanomolar concentrations, high sequence specificity over a thousandfold concentration range, little or no nonantisense activity, total stability in blood and within cells, excellent water solubility, and low production costs relative to other antisense structural types. Our efforts are now focused on achieving effective delivery into the cytosol/nuclear compartment of cells by means suitable for therapeutic applications and on studying the activities of these oligos in animals.

ACKNOWLEDGMENTS

We thank Donald Johnson, Christopher Matthews, Denis Burger, and Alan Timmins for helpful suggestions during the preparation of this manuscript.

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Received January 28, 1997; accepted in revised form March 6, 1997